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Evaluation of a New Continuous Colorimetric Method for Determination of Serum Pseudocholinesterase Catalytic Activity and its Application to a Centrifugal Fast Analyser[1])

By *M. Panteghini* and *R. Bonora*

1st Laboratory of Clinical Chemistry, Spedali Civili, Brescia, Italy

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Summary: We report the evaluation of a new commercially available assay system for the determination of serum pseudocholinesterase (EC 3.1.1.8) catalytic activity, and its application to a kinetic analyser.

The assay is based on the colorimetric method of *Okabe* et al. (Clin. Chim. Acta *80*, 87–94 (1977)): choline, liberated from benzoylcholine by pseudocholinesterase, is oxidized by choline-oxidase (EC 1.1.3.17) to betaine with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a coloured compound with maximal absorbance at 500 nm. The procedure not only has the advantage of being continuous, colorimetric and totally enzymatic but also appears to be precise (between-day analysis gives coefficient of variation between 3.5 and 5.6%) and accurate; the results obtained from normal and pathological sera show excellent correlation with those obtained by the alternative procedures employing propionylthiocholine, acetylthiocholine and butyrylthiocholine as substrates.

Bewertung einer neuen kontinuierlichen kolorimetrischen Methode zur Bestimmung der katalytischen Aktivität von Pseudocholinesterase im Serum und ihre Anwendung an einem Zentrifugal-Analysengerät

Zusammenfassung: Wir berichten über die Bewertung eines neuen, kommerziell erhältlichen Bestimmungsverfahrens der katalytischen Aktivität von Pseudocholinesterase (EC 3.1.1.8) im Serum und ihre Anwendung an einem Zentrifugal-Analysengerät. Die Bestimmung beruht auf der kolorimetrischen Methode von *Okabe* et al. (Clin. Chim. Acta *80*, 87–94 (1977)): Durch Pseudocholinesterase aus Benzoylcholin freigesetztes Cholin wird durch Cholinoxidase (EC 1.1.3.17) zu Betain oxidiert; das dabei gebildete Wasserstoffperoxid koppelt unter Einwirkung von Peroxidase 4-Aminoantipyrin und Phenol oxidativ zu einem Farbstoff mit einem Absorptionsmaximum bei 500 nm. Das Verfahren hat nicht nur den Vorteil einer kontinuierlichen, kolorimetrischen vollenzymatischen Methode, sondern scheint auch genaue (VK von Tag zu Tag 3,5 bis 5,6%) und richtige Resultate zu ergeben. Die bei Untersuchung von Seren Gesunder und Kranker erhaltenen Ergebnisse zeigen eine hervorragende Korrelation mit den Ergebnissen anderer Methoden, bei denen Acetyl-, Propionyl- und Butyrylthiocholin als Substrat verwendet wird.

Introduction

The determination of serum pseudocholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) is commonly requested as a test of hepato-cellular function, to monitor poisoning and excessive exposure to the anticholinesterase organophosphorus insecticides and, mainly, for the detection of patients with atypical forms of the enzyme that could react abnormally with succinylcholine (suxamethonium) employed as a muscle relaxant (1, 2). Indeed, failure of the atypical enzyme to hydrolyze succinylcholine may result in prolonged apnoea in the patient during anaesthesia. Finally, two recent reports (3, 4) have suggested that pseudocholinesterase catalytic activity may be

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important in the assessment of the risk for cardiovascular disease.

Several methods have been developed for the determination of pseudocholinesterase. The numerous colorimetric methods (5), using iodide salts of acetyl (6–8), propionyl (9) and butyrylcholine (8, 10, 11) as substrates, include the procedure of *Ellman* (6) in which the substrates behave similarly to choline esters, and the thiocholine produced by enzymic hydrolysis can be measured by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). In 1977, *Okabe* et al. (12) described a new totally enzymatic approach to determination of serum pseudocholinesterase catalytic activity by estimating choline derived from benzoylcholine using choline-oxidase and a coupled enzyme system. This paper reports the evaluation of a new commercially available assay system, based on this method, and its application to a kinetic analyser. For reference, we chose the method of *Dietz* et al. (9) adopted as a Selected Method by the American Association for Clinical Chemistry, the method of *Garry & Routh* (acetylthiocholine as substrate) (7) and the method of *Das & Liddell* (butyrylthiocholine as substrate) (10). There is no consensus among investigators as to the preferred assay temperature; most determinations have been carried out at 25 °C or 37 °C (13). The International Federation of Clinical Chemistry has recommended that assay at 30 °C should become the standard procedure (14). It therefore seems desirable not only to assess the commercial kit at 30 °C but also to compare the results with those obtained with reference methods at the same temperature (11).

Materials and Methods

Blood samples

Serum containing the homozygous "atypical" (E_1^a E_1^a) enzyme used for this study was obtained from hospital patients who experienced prolonged apnoea when administred succinylcholine in conjunction with surgery. "Heterozygous" (E_1^u E_1^a) and "usual" (E_1^u E_1^u) enzymes were obtained from members of families of those individuals demonstrating the "atypical" enzyme and from patients in the Brescia area. Blood was taken from an antecubital vein and allowed to clot; after centrifugation (1000 *g*, 10 min), the unhaemolysed serum was separated and stored at −20 °C until required. Under such conditions of storage, cholinesterase catalytic activity has been shown to be constant for several months (13).

Measurement of enzyme catalytic activity

The pseudocholinesterase catalytic concentration was expressed in terms of U/l serum (1 Unit = 1 μmol of substrate converted per minute) measured at 30 °C. All the enzymatic determinations were carried out on Cobas Bio centrifugal analyser (F. Hoffmann La Roche and Co., Ltd., Basle, Switzerland). Analyses were performed in triplicate and the mean value was calculated. For the procedures involving the *Ellman* reaction (6), the enzymatic activity was calculated using 1360 $m^2 \cdot mol^{-1}$ as the molar lineic absorbance value for 5-thio-2-nitrobenzoic acid, the product of the reaction.

Propionylthiocholine as substrate

The Selected Method of *Dietz* et al. (9) was used.

Butyrylthiocholine as substrate

Pseudocholinesterase assay using butyrylthiocholine as substrate was performed according to the method of *Das & Liddell* (10).

Acetylthiocholine as substrate

The method of *Garry & Routh* (7) was used.

Dibucaine inhibition

Dibucaine concentrations of 30 μmol/l were used for the acetyl and propionylthiocholine methods; to achieve a similar inhibition with butyrylthiocholine as the substrate, a dibucaine concentration of 54 μmol/l was needed (2). For the method under evaluation, the inhibition curve of pseudocholinesterase by dibucaine was prepared with inhibitor concentrations varying from 30 μmol/l to 5.0 mmol/l. The percentage inhibition was calculated as described previously (9).

Benzoylcholine as substrate

The Enzyfast MA 701 Cholinesterase assay kit (product no. 7019) was a gift from Poli Industria Chimica, Milan, Italy. The assay is based on the method of *Okabe* et al. (12): choline, which is liberated from benzoylcholine as substrate by pseudocholinesterase, is oxidized by choline-oxidase (EC 1.1.3.17) to betaine with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase (EC 1.11.1.7) to yield a coloured compound with maximal absorbance at 500 nm. The molar lineic absorbance for the dye formed under these conditions was 689 $m^2 \cdot mol^{-1}$.

The kit consists of separate reagents for enzymes and chromogen, buffer (tris(hydroxymethyl)aminomethane-hydrochloric acid, 0.2 mol/l, pH 7.5) and substrate. To prepare the working solution, we dissolved the lyophilized enzymes and a tablet of substrate in 20 ml of buffer; the final concentrations of the components in the working solution were 1650 U/l choline-oxidase, 3×10^7 U/l peroxidase, 0.8 mmol/l 4-aminoantipyrine, 30 mmol/l phenol and 1 mmol/l benzoylcholine chloride.

Procedure

Cobas Bio was operated according to the usual protocol, the settings being shown in table 1, unless otherwise stated. Dibucaine inhibition studies were performed by the simple substitution of the reagent containing this material for the usual reagent.

Tab. 1. Parameter listing for determination of pseudocholinesterase catalytic activity on the Cobas Bio centrifugal analyser by the evaluated method.

Units	1 (U/l)
Calculation factor	18142
Standard concentration	0
Limit	1.26
Temperature (°C)	30.0
Type of analysis	2 (reaction rate)
Wavelength (nm)	500
Sample volume (μl)	2
Diluent volume (μl)	30
Reagent volume (μl)	300
Time of first reading (s)	180.0
Time interval (s)	10
Number of readings	21
Blanking mode	1 (reagent blank)
Printout mode	1

Statistical analysis

Calculations of means, standard deviations, coefficients of variation, correlation coefficients and linear regression analysis were carried out by standard methods (15).

Results and Discussion

Determination of pseudocholinesterase catalytic activity

A calibration curve relating serum volume to the hydrolysis of benzoylcholine was prepared, using serum with catalytic concentration of 1978 U/l. The hydrolysis of benzoylcholine was linearly related to the volume of serum up to 3 µl (fig. 1). Investigation of the effect of incubation time (lag time) on the linearity of the enzymatic reaction showed that the best linearity is obtained with an incubation time of 3 min.

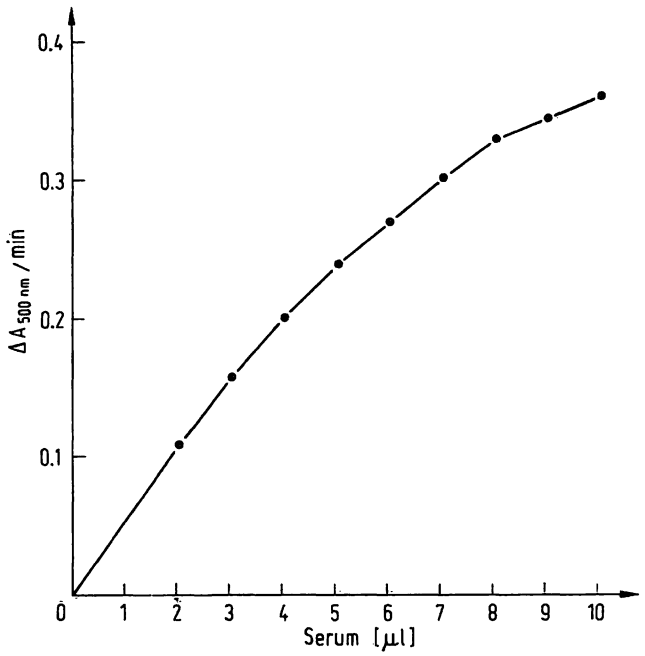

Fig. 1. Effect of amount of serum used in the assay system on the measured pseudocholinesterase catalytic activity.

Reagent stability

The working solution is stable for at least six days if stored at 0—4 °C in a dark bottle.

Linearity

Under the described assay conditions, the reaction is linear up to 6500 U/l of serum pseudocholinesterase ($\Delta A/min = 0.358$); samples with values above this were diluted appropiately in saline with no apparent deleterious effects.

Precision

Precision studies using human sera with low, normal and above-normal catalytic concentrations of pseudocholinesterase are shown in table 2. The coefficient of variation in serial analyses is between 2.9 and 4.6%; between-day analysis of 10 observations for 4 different serum samples gives coefficients of variation between 3.5 and 5.6%.

Tab. 2. Precision of pseudocholinesterase determination by the evaluated method.

Within-run (n = 25)			Between-day (n = 10)		
$\bar{x}$ (U/l)	SD (U/l)	CV (%)	$\bar{x}$ (U/l)	SD (U/l)	CV (%)
227	6.8	3.0	345	12.7	3.7
970	31.2	3.2	690	38.4	5.6
1753	64.6	3.7	1713	92.3	5.4
2023	93.0	4.6	3094	112.1	3.6
6350	181.7	2.9	5010	175.3	3.5

Interference studies

The addition of various kinds of anticoagulant, such as ethylenediaminetetraacetic acid dipotassium salt, sodium citrate, or lithium heparin had no effect on the evaluated assay method. Sodium fluoride, however, is a known inhibitor of pseudocholinesterase (16). Common serum components, i.e. triglycerides, haemoglobin and bilirubin do not interfere up to values of 22.5 mmol/l, 20 g/l and 340 µmol/l, respectively. Reducing substances, i.e. ascorbic acid, glucose, creatinine and uric acid do not interfere up to values of 140 µmol/l, 55 mmol/l, 4500 µmol/l and 1800 µmol/l, respectively.

Comparison with the reference methods

As shown in figures 2, 3 and 4, correlation between the values obtained with the evaluated method and the reference methods was excellent, with correlation coefficients between 0.973 and 0.984.

Reference interval

Since normal pseudocholinesterase values vary with different substrates used (13), we calculated the reference interval for the studied method with respect to the reference methods, employing non-parametric determination of percentiles (17) (tab. 3). Serum pseudocholinesterase levels were determined in 700 healthy people with normal serum biochemical and

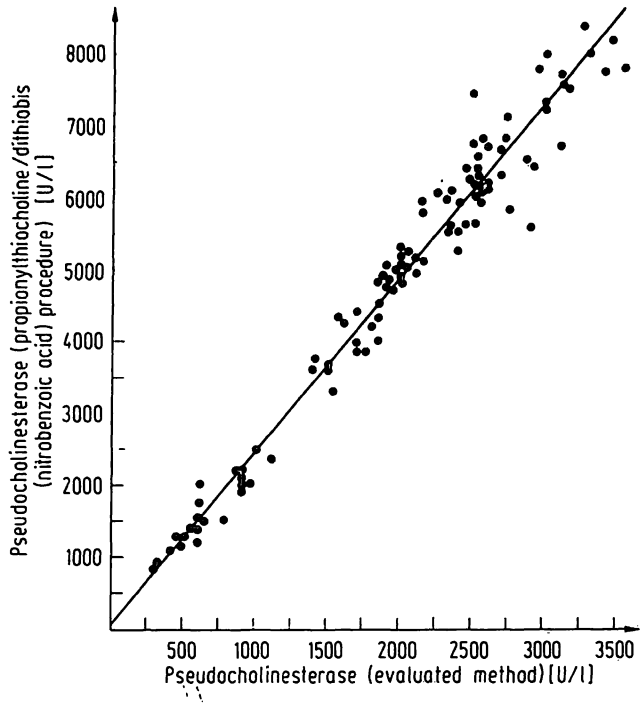

Fig. 2. Correlation between total pseudocholinesterase catalytic concentrations obtained with the evaluated procedure (x-axis) and with the method of *Dietz* et al. (y-axis). Linear regression analysis yields a regression equation of y = 2.41x + 79.45 (solid line) with a coefficient of correlation of 0.980 and a standard error of the estimate of 414.9. Number of patients samples is 100.

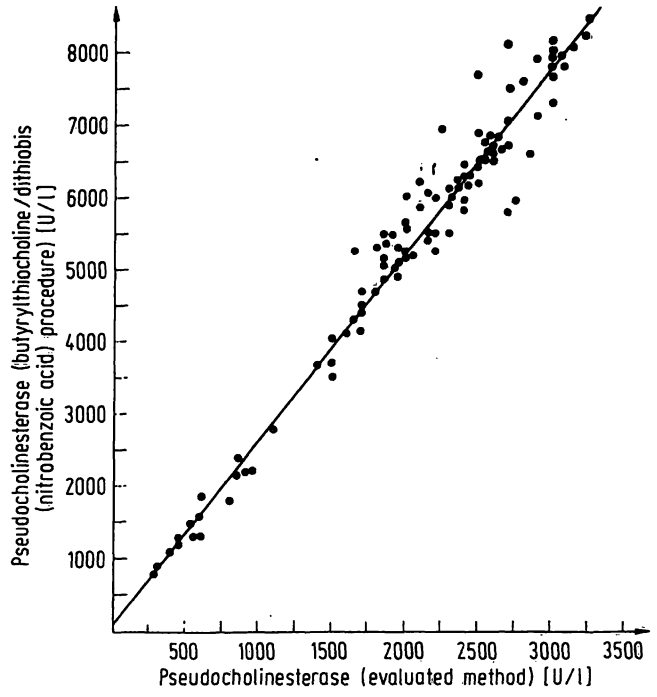

Fig. 3. Correlation between total pseudocholinesterase catalytic concentrations obtained with the evaluated procedure (x-axis) and with the method of *Das & Liddell* (y-axis). Linear regression analysis yields a regression equation of y = 2.58x + 77.12 (solid line) with a coefficient of correlation of 0.984 and a standard error of estimate of 398.8. Number of patients samples is 100.

haematological tests and without clinical evidence of liver diseases (350 women and 350 men, whose ages ranged from 10 to 65 years). The lower reference limit for males is significantly higher than the value for females ($p < 0.001$) (fig. 5); this sex difference has been observed by other investigators (7, 8) and is probably due to lower values in classes of fertile women (18).

Tab. 3. Reference interval for serum pseudocholinesterase catalytic activity concentration measured at 30 °C.

Substrate	2.5th–97.5th percentile (U/l)
Benzoylcholine	1755 to 3760
Propionylthiocholine	3100 to 8670
Butyrylthiocholine	3330 to 9200
Acetylthiocholine	1360 to 3700

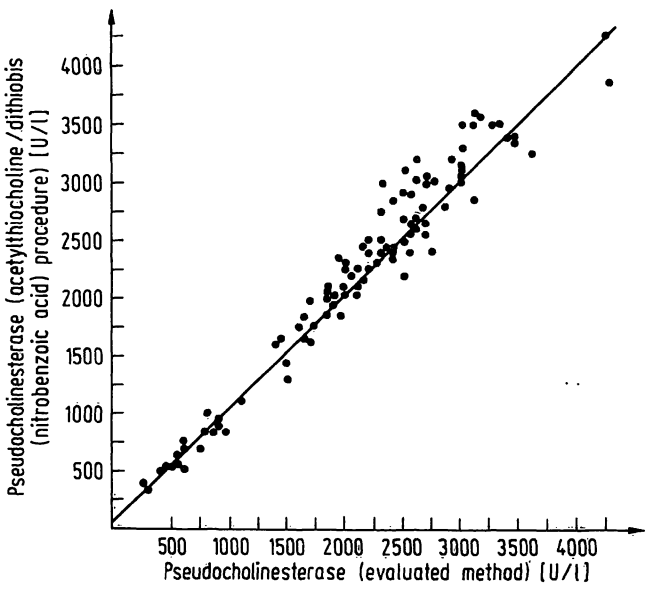

Fig. 4. Correlation between total pseudocholinesterase catalytic concentrations obtained with the evaluated procedure (x-axis) and with the method of *Garry & Routh* (y-axis). Linear regression analysis yields a regression equation of y = 1.01x + 100.50 (sol ' line) with a coefficient of correlation of 0.973 and a standard error of estimate of 203.7. Number of patients samples is 100.

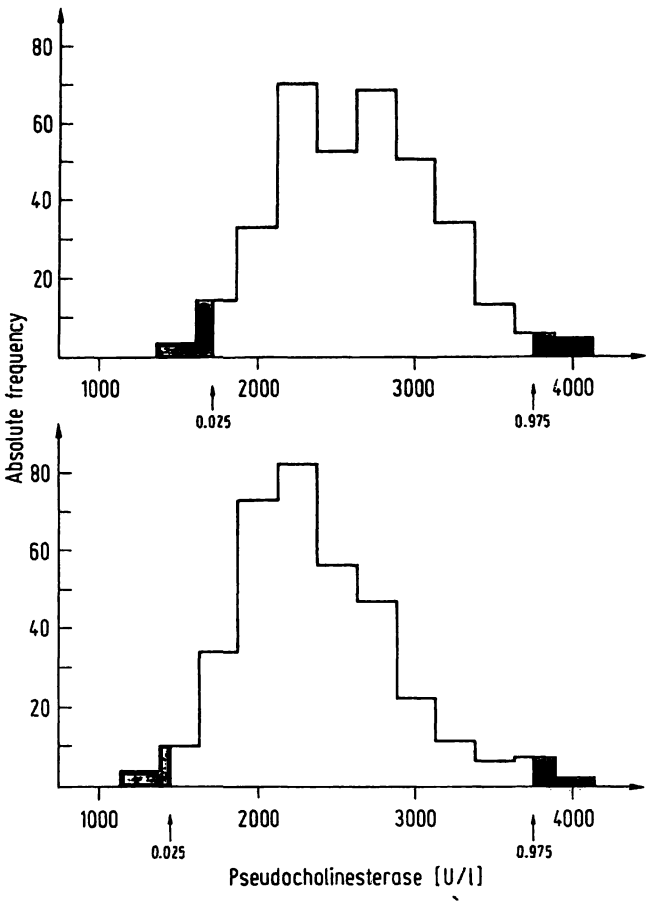

Fig. 5. Histograms showing the frequency distribution of pseudocholinesterase values of 350 healthy males (top) and 350 healthy females (bottom). The arrows point at the 0.025 and 0.975 fractiles (determined non-parametrically: 1850 and 3767 U/l for men, 1645 and 3756 U/l for women, respectively) bounding the central 0.95 fraction of values.

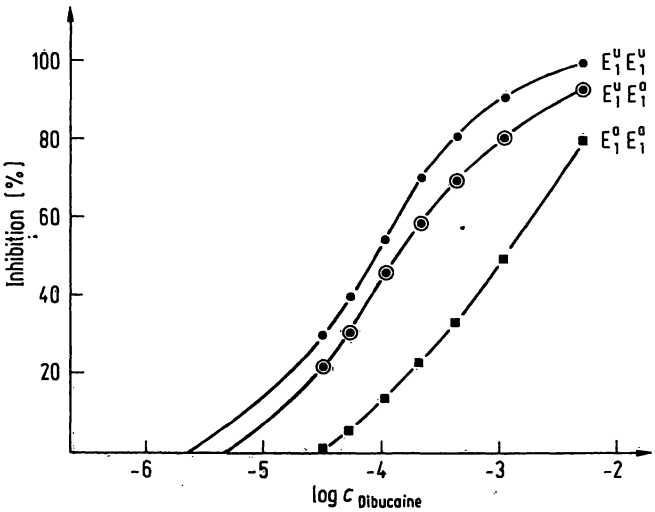

Fig. 6. Curves of the inhibition of "usual" and "atypical" forms of serum pseudocholinesterase by dibucaine (30 µmol/l to 5 mmol/l).

Dibucaine inhibition

Figure 6 shows the inhibition curves of the three pseudocholinesterase phenotype variants as a function of dibucaine concentration. From this figure one can see that the maximum inhibition differences among the three phenotypes are approximately −3.46 log molar dibucaine concentration, which corresponds to 350 µmol/l dibucaine concentration. With this concentration, we reported a mean inhibition of 78.5% (values ranged from 75 to 82%) for 70 subjects whose sera contained the "usual" enzyme, 68% inhibition (values ranged from 45 to 72%) for 20 who had "heterozygous" enzyme and 27% (values ranged from 24 to 29%) for 10 who had "atypical" sera. Regression equations from the experimental data of the comparison of dibucaine numbers, obtained with the enzymic and colorimetric procedures, are presented in figures 7, 8 and 9; in all instances, the enzymic method and colorimetric methods appeared to agree. Therefore, with benzoylcholine and dibucaine inhibition, we could clearly differentiate the genetic variants of pseudocholinesterase; besides, *Kalow & Genest* (19) first demonstrated the ease with which the "usual" enzyme could be distinguished from the "atypical" and "heterozygous" forms of the enzyme, using benzoylcholine as substrate and dibucaine as differential inhibitor.

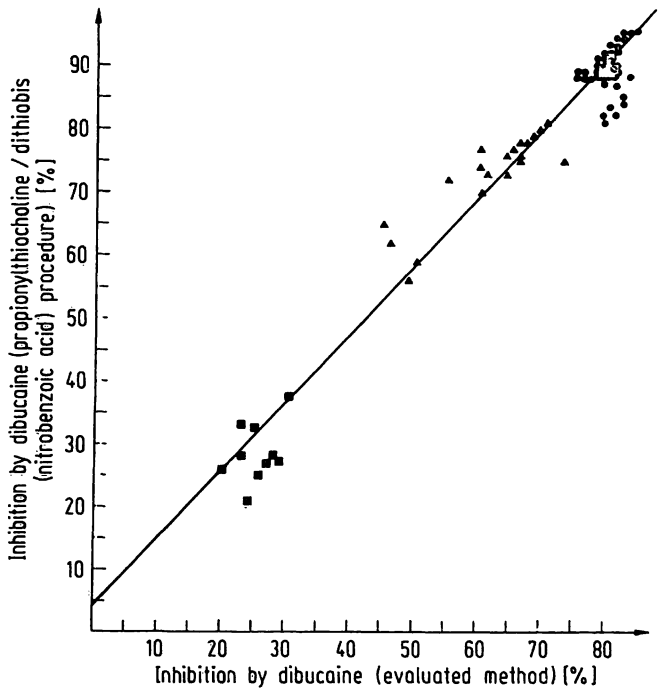

Fig. 7. Correlation diagram for dibucaine numbers (percentage of inhibition) obtained with the evaluated method (x-axis) vs those with the method of *Dietz* et al. (y-axis). The solid line represents the regression equation, y = 1.10x + 4.48, with a correlation coefficient of 0.950 and a standard error of the estimate of 4.93. Solid circle: "usual" enzyme (40 samples); solid triangle: "heterozygous" enzyme (20 samples); solid square: "atypical" enzyme (10 samples).

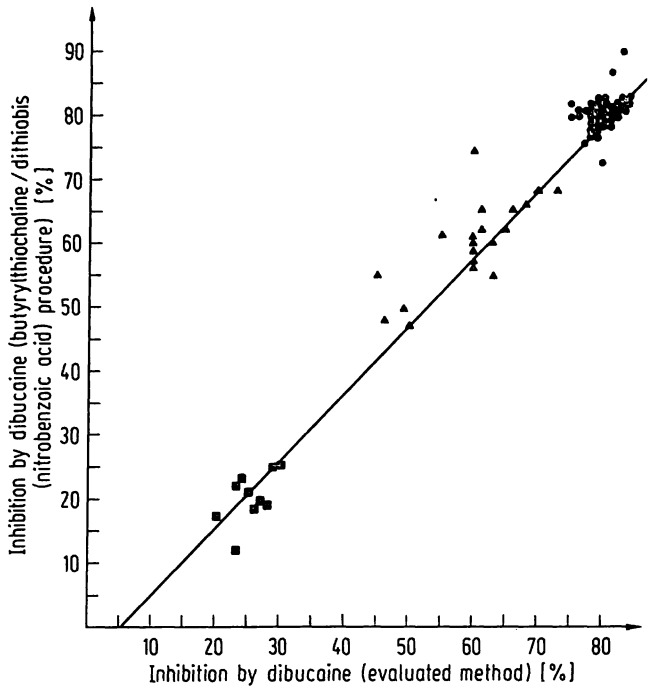

Fig. 8. Correlation diagram for dibucaine numbers (percentage of inhibition) obtained with the evaluated method (x-axis) vs those with the method of *Das & Liddell* (y-axis). The solid line represents the regression equation, y = 1.04x − 5.18, with a correlation coefficient of 0.972 and a standard error of estimate of 3.97. Solid circle: "usual" enzyme (40 samples); solid triangle: "heterozygous" enzyme (20 samples); solid square: "atypical" enzyme (10 samples).

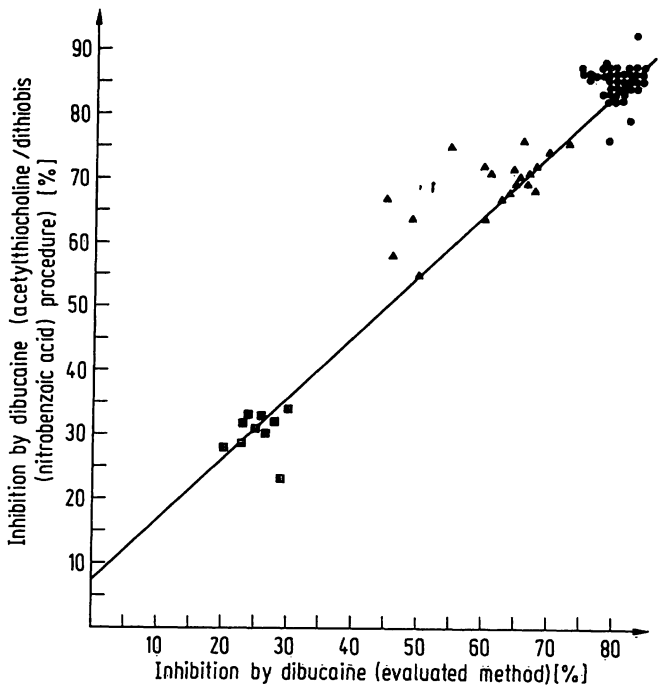

Fig. 9. Correlation diagram for dibucaine numbers (percentage of inhibition) obtained with the evaluated method (x-axis) vs those with the method of *Garry & Routh* (y-axis). The solid line represents the regression equation, y = 0.95x + 7.39, with a correlation coefficient of 0.949 and a standard error of estimate of 5.04. Solid circle: "usual" enzyme (40 samples); solid triangle: "heterozygous" enzyme (20 samples); solid square: "atypical" enzyme (10 samples).

In conclusion, the evaluated procedure not only has the advantage of being continuous, colorimetric and totally enzymatic but also appears to be accurate, precise and relatively simple to perform; its satisfactory performance is indicated by the good agreement between the results of the assay kit and the three reference methods. Finally, this procedure appears to be applicable to several different type of automatic analysers.

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References

1. Whittaker, M. (1980) Anaesthesia *35*, 174–197.
2. Garry, P. J. (1971) Clin. Chem. *17*, 183–191.
3. Kutty, K. M., Jain, R., Huang, S. & Kean, K. (1981) Clin. Chim. Acta *115*, 55–61.
4. Jain, R., Kutty, K. M., Huang, S. & Kean, K. (1983) Clin. Chem. *29*, 1031–1033.
5. Evans, R. T. & Wroe, J. (1978) Clin. Chem. *24*, 1762–1766.
6. Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, R. M. (1961) Biochem. Pharmacol. *7*, 88–95.
7. Garry, P. J. & Routh, J. I. (1965) Clin. Chem. *11*, 91–96.
8. Szasz, G. (1968) Clin. Chim. Acta *19*, 191–204.
9. Dietz, A. A., Rubinstein, H. M. & Lubrano, T. (1973) Clin. Chem. *19*, 1309–1313.
10. Das, P. K. & Liddell, J. (1970) J. Med. Genet. *7*, 351–355.
11. Whittaker, M., Britten, J. J. & Dawson, P. J. G. (1983) Clin. Chem. *29*, 1746–1751.
12. Okabe, H., Sagesaka, K., Nakajima, N. & Noma, A. (1977) Clin. Chim. Acta *80*, 87–94.
13. Kachmar, J. F. & Moss, D. W. (1976) In: Fundamentals of clinical chemistry (Tietz, N., ed.), 2nd ed., pp. 643–649, W. B. Saunders Co., London.
14. Haeckel, R., Hørder, M. & Zender, R. (1982) J. Clin. Chem. Clin. Biochem. *20*, 947–958.
15. Armitage, P. (1971) Statistical methods in medical research. Wiley, New York.
16. Garry, P. J., Owen, G. M. & Lubin, A. H. (1972) Clin. Chem. *18*, 105–109.
17. Reed, A. H., Henry, R. J. & Mason, W. B. (1971) Clin. Chem. *17*, 275–284.
18. Sidell, F. R. & Kaminskis, A. (1975) Clin. Chem. *21*, 1393–1395.
19. Kalow, W. & Genest, K. (1957) Can. J. Biochem. Physiol. *35*, 339–346.

Dr. Mauro Panteghini
1st Laboratory of Clinical Chemistry
Spedali Civili
I-25100 Brescia